Foliar loading and metabolic assimilation of dry deposited nitric acid air pollutants by trees

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Dry deposition of nitric acid vapor (HNO_3) is a major contributor to eutrophication of natural ecosystems. Although soil fertilization by nitrogen deposition is considered to be the primary pathway for changes in plant nutrient status and shifts in ecological structure, the aerial portion of plants offer many times the surface area in which to collect atmospheric HNO_3. As much as 60% of deposited nitrogen may be retained in the canopy and not land on the soil surface below. Although uptake and assimilation appears to contribute to retention, only a small percentage of dry deposition is recovered in assimilated N pools. To test the importance of biological activity on the process and measurements of dry deposition, we used controlled environmental chambers to compare deposition to living and freeze-dried foliage of four tree species using ^{15}N-labeled HNO_3. In living trees, assimilation was determined by ^{15}N incorporation into free amino acids and proteins in leaves and roots. From 10% to 60% of the retained HNO_3 was incorporated into the biologically active nitrogen pool. The remainder was bound to foliar surfaces in an insoluble form in either living or freeze-dried foliage. The importance of the boundary layer conditions emerged as a primary factor controlling dry deposition characteristics and measurements.
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Introduction

Nitric acid vapor is a secondary air pollutant common to urban areas throughout the world. It is synthesized from the same volatile organic carbon and nitrogen oxides precursors, and by the same photochemical processes, that form ozone.[1] Real time ambient measurements of nitric acid (HNO_3) independent of other nitrogen oxides is not yet feasible for monitoring purposes, but extrapolation from ozone measurements indicates a diurnal pattern of very low HNO_3 concentrations at pre-dawn, followed by an increase in concentration with sunrise and peaking at roughly midday. Using this pattern, a 12 h average concentration as determined by denuders systems[2] of 2.0 ppb (w/v) (5.1 μg m^{-3}) would have peak midday concentrations of 35 to 40 ppb.[3] Twenty-four hour HNO_3 concentrations of 1 ppb are a generally accepted threshold for excessive pollution, although no regulatory standard exists in the US.[1] Twelve hour average concentrations of 2 ppb are not unusual in polluted environments, and daytime average concentrations up to 10 ppb (27 μg m^{-3}) have been recorded in the mountains of southern California.[4]

Once formed, HNO_3 generally does not participate in further gaseous phase reactions, but becomes quite reactive with aqueous and solid phase substrates. Nitric acid readily solubilizes in water droplets, it adheres to dust particles, participates in the formation of aerosols, particularly ammonium nitrate, and interacts directly with exposed surfaces. The deposition velocity of HNO_3 is among the highest of the atmospheric gases; the residence time is usually less than a week.[5,6,1] Atmospheric deposition of HNO_3 occurs in wet forms as rain, snow, or cloud condensation, and in dry forms as molecular HNO_3 or particulate-containing NO_3^-. Of the two, dry deposition has been the most difficult to study and quantify.

In arid and semi-arid regions, such as the southwestern United States and Mediterranean Europe, dry deposition of pollutants dominates the depositional processes. In southern California estimates of terrestrial loading by atmospheric nitrogen (N) pollutants are in the range of 35 to 50 kg N per hectare annually. Of that, between 80 to 95% occurs in the dry form at lower elevations and as a combination of dry and fog deposition in mountain environments.[4,7] In the temperate regions of the US, dry deposition may contribute as much as 50% of the total terrestrial load, but dry deposition is even more difficult to measure when combined with frequent wet deposition events.[8,9]

One of the simplest methods of estimating dry deposition to ecosystems is to use the native vegetation as passive collectors. Throughfall studies take advantage of periodic rain events to rinse foliage of accumulated dry deposition. The rainwater that passes through the canopy is collected from beneath the canopy and compared to rainwater collected from an open site. When the throughfall N concentrations are greater than the N concentration in rainwater the difference is attributed to dry deposition.[9,10] Leaf wash studies use the same principles, but rather than relying on rain, leaves are deliberately washed prior to the exposure to dry deposition, and then again after some period of time.[11,12]

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The solution from the second washing is used to estimate the quantity of N deposited over the exposure time.

Although conceptually simple, estimating terrestrial loading from dry deposition using throughfall or leaf wash methods has large associated uncertainties.[13] Calculated dry deposition values vary widely from stand to stand within the same forest or vegetation type, and can vary from tree to tree within the same stand.[8,9,14] The large differences in deposition characteristics to different plant species limit the ability to compare deposition values across ecosystems.[15,16] It is, however, these differences in dry deposition behavior among plant species, forest stands, and ecosystems that suggest an important direct interaction between the plants and atmospheric deposition.

Several authors have used the term 'canopy retention' to identify some of the interactions between deposition and plant biology. The percentage of deposition retained by a canopy can be surprisingly high. Schultz[17] reported that 41% to 63% of the deposited N was retained in spruce canopies, and Friedland *et al.*[18] estimated that 30% of the dry N-deposition was retained by spruce-fir forests. Where and how the 30% to 60% of the deposited N gets trapped in the canopy is not known, but several factors impinge on retention rates. Large differences in retention characteristics between evergreen and deciduous species, occur and large differences even within the conifer family occur.[19,20] In general, hardwoods tend to retain more N than conifers, but as live foliage is present for only part of the year, the available surface area changes dramatically from season to season.[19] Atmospheric concentrations during the exposure period and time between rainfall events are also important factors controlling apparent retention.[13] Dasche[21] and Cadle *et al.*[22] pointed out that deposition velocities of HNO_3 decreased with increased exposure under experimental conditions, and leaves exhibited a maximum deposition load, equivalent to surface saturation. Finally, uptake and assimilation, or 'consumption', of deposited N contributes to canopy retention,[23] but the percent of dry deposition recovered as metabolites is generally low, typically 1% to 5% of the amino acids or proteins.[24]

Air pollution studies designed to understand depositional fluxes of HNO_3, and the uptake and assimilation mechanisms of atmospheric HNO_3 often have employed ^{15}N as a tracer.[22,24–26] Because of earlier methodological problems, fumigations were generally of short duration and at higher atmospheric concentrations than under ambient conditions.[25] But, from these studies several important principles have been established:

- Boundary layer resistance to surface deposition is very low.[27]
- Deposition velocities to a single surface changes with time.[21]
- Calculated deposition velocities vary widely across plant species.[28]
- Cuticle characteristics strongly affect deposition characteristics.[19]
- Uptake by foliage of dry deposited HNO_3 occurs both through the cuticle and by stomatal conductance.[22,29]
- Once in the apoplast, HNO_3 is incorporated into the biologically active nitrogen pools.[24,25,30]

The work reported here was undertaken to build on the earlier foundations, and to address some of the lingering issues relating atmospheric processes to ecological effects.[19]

A continuously stirred tank reactor (CSTR) system developed for HNO_3 fumigation[3] allowed us to expose ponderosa pine (*Pinus ponderosa*, Laws), white fir (*Abies concolor*, Lindley), canyon live oak (*Quercus chrysolepis*, Liebm.) and California black oak (*Quercus kelloggii*, Newb.), in a controlled environment to zero, moderate, and high levels of HNO_3 vapor using diurnal patterns and concentrations that reflect the ambient conditions in southern California. The chambers enabled us to conduct experiments for one month or more, and to sample foliage repeatedly during the experiment. We used ^{15}N-labeled HNO_3 vapor to quantify dry deposition, foliar retention, and assimilation of absorbed NO_3^- into free amino acids and soluble proteins isolated from leaf and root tissue. To evaluate the effects of biological activity on dry deposition, experiments were conducted comparing deposition to living foliage and to foliage that was harvested from the same individuals but was freeze dried. The objectives of these studies were to understanding the sources of variability in deposition rates to different tree species, the chemical, physical and biological basis of canopy retention, and to quantify assimilation of dry deposited HNO_3.

Experimental

Species and growth conditions

Four tree species native to forests of the western United States were evaluated. Each of the four represents a different growth pattern, or ecological niche[31] Ponderosa pine is the dominant species in western mixed coniferous forests at elevations below 2500 metres. It is relatively long-lived and fire resistant at maturity. White fir is often an understorey species in mixed conifer forests. It is shade tolerant but not fire resistant. It can be found in monotypic stands where moisture availability is relatively abundant such as north facing slopes and riparian corridors. California black oak is also an understorey species. It is deciduous and may grow as a medium sized single-trunk tree or a large multi-trunk shrub. It resprouts readily after fires and will often dominate sites after fires. California black oaks are only moderately shade tolerant and will decline in mixed conifer forests as the canopy closes. Canyon live oak is among the most adaptable of western tree species. It is an evergreen oak that grows in many forms from statuesque specimens to multi-trunk shrubs to semi-creeping forms. It is drought tolerant, often inhabiting dry south-facing slopes, but it will also occupy riparian zones. Canyon live oak is often a component of chaparral vegetation as well as a component of forests.

The white fir and ponderosa pine seedlings were obtained from the California Department of Forestry Reforestation Nursery (Davis, CA, USA). They were shipped as bare-root, 2 year-old seedlings. They were potted into 7 litre pots and had completed the annual growth cycle prior to the fumigation experiments. The California black oak seedlings were half siblings, transplanted from the field in the San Bernardino Mountains, in southern California. They were three years old at the time of the experiment. The canyon live oak seedlings were grown from acorns collected under an isolated tree on the University of California, Riverside (UCR), campus. They were also three years old. All seedlings were grown in the greenhouse where the fumigation facility was housed. One month prior to beginning the experiment, 5 grams of slow-release fertilizer (Osmocote®, The Scotts Co. Marysville, OH, USA) were added to the pots to maintain

soil nutrient levels. The leaves or needles were fully expanded, and the conifers were initiating bud set for the next year's growth.

The pots were not protected from direct deposition to the soil surface, but were instead watered from below. Earlier studies have demonstrated that dry deposited HNO_3 remains on the surface of soils and does not migrate into the soil profile until watered from above.[32]

Freeze drying of foliage for testing biological activity effects

One week prior to the fumigations, six leaves from each oak tree, six fascicles from each pine tree, and six branchlets from each fir tree were clipped, frozen in liquid nitrogen, and lyophilized. An artificial tree was then reconstructed for each individual matching the dead leaves to their living companions. The artificial tree consisted of a 10 cm column of rigid Styrofoam, 2 cm by 2 cm in cross section. Leaves were inserted into the Styrofoam at the same height as the living foliage, and the column was pegged into the pot along side of the living tree.

Fumigation system and protocols

Fumigations were carried out in constantly stirred tank reactors (CSTR) describe by Padgett *et al.*[3] The fumigation protocols were designed to mimic pollutant diurnal patterns and concentrations typical of high and moderately high pollutant loads in southern California. The CSTRs were 1.5 metres tall and 1 metre in diameter, and were made of a clear Teflon sheet surrounding a Teflon frame. The chambers were capable of holding twenty 7 litre pots. To achieve HNO_3 vapor, 10% (v/v) aqueous $H^{15}NO_3$ (98% atom mol) was introduced into a volatilization chamber heated to 84 °C. Ambient air was dried using a heatless air dryer to less than 1% relative humidity and passed through charcoal and HEPA filters before being introduced to the volatilization chamber. Volatilized HNO_3 and evaporated water moved into a glass manifold and was delivered to each CSTR separately *via* glass and Teflon tubes. The concentrations in the individual chambers were regulated by the rate at which liquid HNO_3 was introduced into the volatilization chamber and by control valves at each fumigation chamber. The entire system was controlled by a set of timers so that it was off during the dark hours and began vapor delivery with sunrise.

Nitric acid concentrations in each chamber were continuously monitored using a standard Nitrogen Oxide Monitor (Thermo Environmental Instruments, Franklin, MA, USA). Because HNO_3 is unstable, molybdenum converters were installed just outside of each chamber in the monitoring air stream so that the air sample is immediately reduced to NO. Nitrogen oxides other than HNO_3 were measured inside and outside the chambers to control for contamination and the HNO_3 concentrations in the chamber were calculated by difference.[3]

To begin the experiment, all leaves of the seedlings were washed gently with deionized distilled (DDI) water to remove dust and other debris. Fifteen morphologically matched seedlings from each species were chosen and five replicates were placed in each of three chambers, a control chamber (receiving only charcoal-filtered air), a moderate concentration (35 ppb peak) HNO_3 chamber, and a high (50 ppb peak) HNO_3 concentration chamber. The cumulative doses for each treatment chamber are shown in Table 1. Dose was calculated by integration of the concentration curves.

Table 1 Cumulative atmospheric concentrations (dose: ppb h) of the two treatment chambers. Dose was calculated by integration of the concentrations over time

	Chamber 3	Chamber 4
14 day	137	161
28 day	262	277

NO_3^- removed—deposition sampling

Dry deposition was determined by the leaf wash method after 14 d and 28 d. Three leaves or needle samples were collected from each individual, living or freeze dried. Leaf samples were placed in a 50 ml conical, disposable, centrifuge tube. Ten ml DDI water were adding, and the tubes were capped and shaken gently for 30 s. Foliage samples were removed, and the wash solutions were analysed by ion chromatography (Dionex Corp. Sunnyvale, CA, USA) for NO_3^-. The dry deposition, or 'N removed' are expressed in terms of μg N cm^{-2}.

The leaf area for each foliage sample was determined immediately after collection or washing using a portable, leaf area meter (model LI-3000, Li-Cor Bioscience, Lincoln, NE, USA). Conversion of leaf area to dry weight was conducted mathematically by equations developed empirically from 100 samples per species. All plant tissue handling was conducted using latex gloves with frequent changes, and the belts of the leaf area meter were washed carefully between samples to reduce the possibility of ^{15}N carry over and contamination of samples.

NO_3^- retained—^{15}N analysis

Following the leaf wash procedures, all live leaf samples were oven dried at 40 °C and ground to a fine powder in liquid nitrogen with a mortar and pestle. Approximately 5 mg of tissue were loaded into a tin capsule; the exact weight was recorded, and the samples were sent to the University of California, Davis stable isotope facility for determinations of ^{15}N enrichment of total N. Foliar retention of deposited ^{15}N was calculated on a dry weight basis from the recorded weights submitted to the laboratory for isotope analysis.

Tissue sampling—amino acids and proteins

We assumed no isotopic discrimination of ^{15}N incorporation by the assimilatory enzymes. However, studies have suggested that isotope discrimination in plant metabolism may be more complicated than initially believed.[33] The ^{15}N content of the HNO_3 was 98%, but we did not correct for the 2% ^{14}N in our calculations as it increased potential error without improving the interpretation of the data.

After 28 d, all living individuals were harvested. Foliage was removed from stem tissue, and the below ground portion of the trees was separated at the soil line. For the conifers and the evergreen live oak, only current year foliage was collected; all leaves were collected from the deciduous black oaks. Roots were washed to remove visible soil particles, and the fine roots were

separated from coarse roots. Only fine roots were used for ^{15}N analysis. Leaf and root samples, as well as stems and woody roots, were frozen intact in liquid nitrogen as they were harvested. Samples were stored at −80 °C prior to lyophilization.

Soluble protein was extracted from lyophilized leaf and fine root tissues in a Peterson[34] Mes-Tris buffer using a PolyTron® (Brinkman Instruments, Westbury, NY, USA) tissue grinder. Approximately 0.1 g of leaf tissue or 0.2 g of root tissue (actual weights were recorded) was cut into smaller pieces and placed into centrifuge tubes, 20 mL of buffer were added, and the sample was ground to fine a suspension. Samples were centrifuged at 7741 relative centrifugal force (RCF) for 20 min, the supernatant was transferred to a second centrifuge tube and the centrifugation repeated to remove insoluble material. The supernatant was decanted, and 1.9 mL of 72% trichloroacetic acid (TCA) were added to precipitate the protein. The samples were then refrigerated overnight. Following refrigeration, the samples were centrifuged at 12096 RCFs for 20 min to separate the precipitable protein from the remaining solution. The pellets were washed carefully with DDI water and dried in an evaporative drier. The dried samples were transferred into tin capsules, and sent to the UC Davis Isotope Analysis facility for determination of ^{15}N enrichment of N content. We recognized that TCA does not precipitate all forms of cellular proteins, particularly proteins embedded in cell membranes and that N is a component of many nonproteinaceous molecules, such as nucleic acids, neither of which were measured. However, as the objective of the experiment was to capture the extent of overall assimilation, we chose to leave the detailed cell fraction to a later study.

Amino acids were isolated using standard ion exchange methods.[35] Approximately 0.2 g leaf tissue or 0.3 g fine root tissue were chopped into small fragments and weighed into a glass test tube. The exact weights were recoded, and 4.0 mL of 80% ethanol were added. The tubes were placed in a boiling water bath, and the ethanol, brought to a boil for 30 s to remove any residual enzyme activity. The extract was transferred to a fresh tube, and an additional 5 mL of ethanol were added to each sample. The samples were placed in a 65 °C water bath for approximately 2 h. This extract was added to the first aliquot, and the procedure was repeated until no more color could be removed from the samples. The tissue samples were discarded. The ethanol extracts were dried in an evaporative drier. The resulting pellet was resuspended in 2 mL of DDI water prior to isolation of amino acids by ion exchange. Ion exchange columns were prepared by adding 1 mL of Dowex AG® 1-X8 (Dow, Midland, MI, USA) anion exchange resin into disposable ion exchange columns. The resuspended pellet was transferred to the column in two portions allowing each to fully enter the resin bed. The resin bed was washed in a total of 20 mL of DDI H_2O in two portions, removing soluble sugars. Amino acids were eluted from the columns using two 1 mL aliquots of 50% acetic acid (8.7 M). The eluent was dried, in an evaporative drier and the resulting pellet was transferred into tin capsules for ^{15}N analysis.

Experimental design and data analysis

The experiment was conducted in a controlled environment by a completely randomized design as described by Potvin.[48] Each CSTR chamber was analysed as an independent treatment as is typical of chamber studies since true replication across chambers is rare.[49] The experimental units were individual trees and each species was replicated five times in each of the treatment chambers. Strictly speaking, the analysis can only separate differences among the chambers as the treatments themselves were not replicated. However, 30 years of research conducted in fumigation chambers have shown that the assumption that the differences among chambers are due to imposed treatments is reasonable.[36]

The 'nitrogen removed' data were log transformed to fit the assumption of normal distribution and analysed by ANOVA. 14 d and 28 d samples were analysed separately by 3-way ANOVA, by HNO_3 treatment level, by species, and by live/dead (freeze-dried). Species responses within treatments were tested using 1-way ANOVA. Post-hoc comparisons were conducted using Student–Newman–Keuls method for multiple pairwise comparisons. Significance was determined by $P < 0.05$. Comparisons across 14 d and 28 d samples, within treatments and species was conducted by Kruskal–Wallis one way ANOVA on ranks and Student–Newman–Keuls pairwise multiple comparison procedures. The '^{15}N retained' data was analysed by one and three way ANOVA using similar procedures described for N removed above. Data analysis of total ^{15}N retained evaluated the response to treatment, the difference among species, and the difference between live and freeze dried plant material, within treatments. Analysis of incorporated ^{15}N (protein and amino acids) was conducted by one-way analysis of treatment levels within species. Significance was determined by $P < 0.05$. All analyses were conducted with SigmaStat 2.0 (Jandel Scientific Software, San Rafael, CA, USA), using a mixed model.

Results

NO_3^- removed from leaves

The data shown in Table 2 are the concentrations of NO_3^- in wash solutions from leaves harvested from three treatment chambers after 14 d or 28 d of exposure. Note that the data is expressed in terms of μg N cm^{-2} rather than NO_3^-, which will enable better comparisons with the ^{15}N retained data later. In all cases total N washed from HNO_3-treated foliage was significantly greater than from control (0 HNO_3 treatment) foliage. Most plants leach salts as part of the transpirational stream, so small quantities of NO_3^- in leaf-wash solutions from untreated leaves are common.

14 day exposures. Examining the data from the wash solutions taken after 14 d exposures to HNO_3 reveals no significant difference in wash-solution concentrations between the two treatment levels, high or moderate, for any of the four tree species. There were no significant interactions between species, HNO_3 level, or live *vs.* freeze-dried foliage. Significant differences among the four tree species within each of the treatment chambers did occur. Ponderosa pine consistently shed more soluble NO_3^- that any of the other species in either the live or freeze-dried category. However, there is less than a 50% difference in concentrations of N on an area basis among the four tree species in the moderate treatment. In the high treatment, ponderosa pine ranked highest in N removed at 1.8 μg N cm^{-2}, among the living

Table 2 NO_3^- (expressed in terms of N) removed from living and freeze dried foliage at two time points. Data shown are the means of 3 samples per individual and 5 individuals per sampling time. Superscript notation indicates separation of means within treatment, live *vs.* freeze dried, and sampling time. Differences between N removed between live and freeze dried foliage are indicated with *

		14 day mean/μg N cm^{-2} (σ)			28 day mean/μg N cm^{-2} (σ)		
HNO_3 level	species	Live foliage	Freeze dried	Difference $P < 0.05$ (freeze dried − live)	Live foliage	Freeze dried	Difference $P < 0.05$ (freeze dried − live)
High	Black oak	1.27 *0.26* [a]	2.34 *0.42* [b]	1.08*	1.16 *0.23* [a]	4.11 *1.06* [a]	2.95*
	Live oak	0.70 *0.11* [a]	2.63 *0.58* [b]	1.93*	0.79 *0.23* [a]	3.65 *0.64* [a]	2.86*
	White fir	0.63 *0.36* [a]	0.68 *0.20* [c]	0.05	1.26 *0.30* [a]	1.94 *0.47* [a]	0.68
	Ponderosa pine	1.94 *0.20* [a]	5.98 *0.69* [a]	4.04*	2.47 *0.33* [a]	6.08 *0.97* [a]	3.62*
Mod	Black oak	1.41 *0.19* [a]	2.05 *0.59* [b]	0.63	1.25 *0.29* [b]	2.58 *0.74* [a]	1.32
	Live oak	1.02 *0.59* [b]	0.92 *0.11* [c]	−0.10	0.69 *0.24* [b]	6.05 *1.48* [a]	5.36*
	White fir	1.04 *0.51* [b]	1.60 *0.78* [c]	0.56	1.12 *0.19* [b]	2.98 *1.10* [b]	1.86*
	Ponderosa pine	1.79 *0.55* [a]	3.85 *0.83* [a]	2.06*	1.54 *0.27* [a]	5.07 *0.76* [a]	3.52*
Control	Black oak	0.23 *0.04*	0.31 *0.05*	0.08	0.22 *0.04*	0.26 *0.05*	0.04
	Live oak	0.19 *0.06*	0.14 *0.02*	−0.05	0.10 *0.01*	0.21 *0.03*	0.10
	White fir	0.45 *0.11*	0.21 *0.04*	−0.25	0.16 *0.05*	0.45 *0.13*	0.28
	Ponderosa pine	0.27 *0.06*	0.19 *0.03*	−0.08	0.14 *0.06*	0.21 *0.07*	0.07

individuals; black oak ranked second at 1.4 μg N cm^{-2} in quantity of N removed, and live oak and white fir were nearly identical with 1.02 and 1.04 μg N cm^{-2} removed.

The quantity of N removed from freeze-dried foliage differed across species. The rankings remained the same as in the live foliage with ponderosa pine being the highest at nearly 6.0 μg N cm^{-2} in the high treatment and 3.8 μg N cm^{-2} in the moderate treatment; black oak was consistently lower than pine (2.3 and 2.0 μg N cm^{-2}) and was greater than white fir (0.7 and 1.6 μg N cm^{-2}), but the ranking with live oak was treatment dependent at 2.6 μg N cm^{-2} in the high treatment and 0.9 μg N cm^{-2} in the moderate treatment.

In comparing N removed from freeze-dried foliage to that removed from live foliage within each species, ponderosa shed two to three times more NO_3^- from the non-living, freeze-dried needles than did their living counterparts. In the high treatment, both the live and black oak lost significantly more soluble NO_3^- from non-living foliage than from live foliage. The difference between live and freeze-dried needles in white fir was not significant for either treatment.

28 day wash solutions. Pairwise comparisons between N removed after 14 d and after 28 d, in general, did not differ in living foliage, but the increase in N removed from non-living, freeze-dried foliage after 28 d was significantly greater than N removed at the 14 d sampling point for most species under high or moderate N treatments.

After 28 d exposure to HNO_3, NO_3^- washed from living foliage was statistically similar across tree species and treatment level. Only ponderosa pine in the moderate treatment was significantly different from the other species. In freeze-dried tissue, only one species-treatment combination differed significantly from the others on a surface area basis—white fir in the moderate treatment was lower than any of the other species/treatment combinations.

In all but white fir from the high treatment, NO_3^- removed from freeze-dried leaves was 2 to 4 times greater than NO_3^- removed from living foliage (Fig. 1). Note that the relationship among the four species changes when N removed was calculated on a dry weight basis as shown in Fig. 1 as compared to a leaf area basis as shown in Table 2. In Fig. 1, the amount of N removed from black oak, living or non-living, is significantly greater than from either white fir or ponderosa pine, in contrast to the data shown on an area basis, where ponderosa pine ranked highest in N removed.

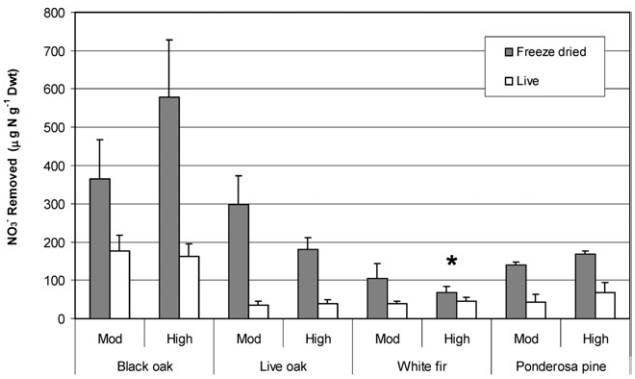

Fig. 1 Dry NO_3^- removed from 4 tree species exposed to HNO_3 vapor for 28 d. Each pot contained a living tree and an artificial tree constructed from freeze-dried leaves from the same individual. Data shown calculated on a dry leaf-weight basis. Data are the means of 3 leaves sampled from 5 individuals. Error bars = σ.

^{15}N tracer studies: NO_3^- retained

Total N. The relationship between ^{15}N retained in leaves (Fig. 2) and N removed by washing of leaves (Fig. 1) was inversely proportional. In all cases the freeze-dried tissue retained significantly less (20% to 80%) ^{15}N than did the living tissue. Living tissue from black oak or live oak retained roughly four times more deposited N than did the conifers on a dry weight basis. But when analysed on a *leaf area* basis (inset Fig. 2), the differences among the species for N-retention were significant only for live oak, which retained more ^{15}N than the other species. Retention of ^{15}N by the freeze-dried leaves was the highest in live oak where retention was nearly three-quarters the value for the

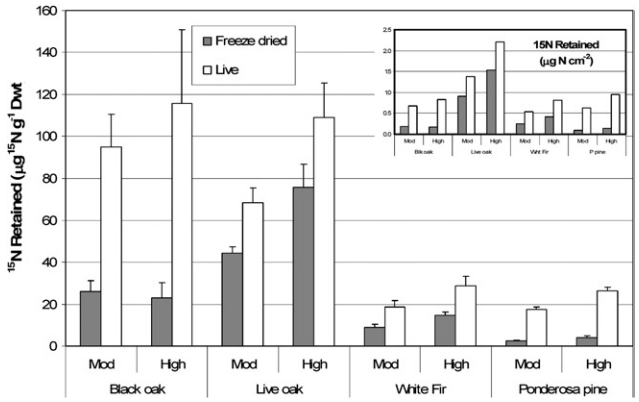

Fig. 2 Total ^{15}N remaining in leaf tissue after washing. Data are the means of 3 leaves or needle groups from 5 individuals. Error bars = σ.

living leaves. In comparison, retention in freeze-dried black oak leaves was approximately 25% of the retention measured in living leaves. A trend for increased retention with increased dose was evident in the living tissue, but the statistical separation was significant only at the $P = 0.1$ level. No significant differences occurred between treatment levels for ^{15}N retained with freeze-dried foliage. Freeze-dried ponderosa pine needles retained the least amount of dry deposited HNO_3. On average, only slightly more ^{15}N was measured in needles from the HNO_3 chambers than in needles from the 0 NO_3 chambers (data not shown) and those differences were not significant ($P < 0.05$).

Uptake and assimilation of deposited HNO_3. The ^{15}N content in the soluble protein pool increased with increasing dose, particularly in the oak species (Fig. 3A). A trend for increased ^{15}N in leaf proteins with increased dose was evident in the conifer species, but the differences between treatments were significant only when compared to the controls which received no HNO_3, but not between the high and moderate treatments. No differences between high and moderate HNO_3 treatments were reflected in the leaf amino acid (AA) pool (Fig. 3B). On a dry weight basis the ^{15}N in AA and proteins are remarkably similar across the four tree species, particularly as compared to the differences in total retained ^{15}N shown in Fig. 2.

Extraction of protein and AA from fine root tissue was not quantitative enough to permit calculation of a weight-based ^{15}N content. Translocation is expressed in terms of change in ^{15}N concentrations relative to controls (Fig. 4). The AA pool was more highly labeled than the protein pool, but the AA pool size was much smaller. The ^{15}N content in live oak was between 50% and 80% greater in treated root tissue than in the controls, and the protein content was between 20% and 30% higher. The smallest change in root ^{15}N content was found in white fir.

One percent or less of the leaf AA pool was labeled after 28 d of dry deposition of HNO_3 vapor (Table 3). The labeled pool of proteins was about half of that. The conifers exhibited lower assimilation rates of foliar-deposited HNO_3 than did the oaks. Since the root extracts were not quantitative enough to allow for a similar calculation, the percentage of ^{15}N in AAs and proteins is unknown, but the data suggest that the percentage is likely to be much lower than the observations for foliar tissue (Fig. 4).

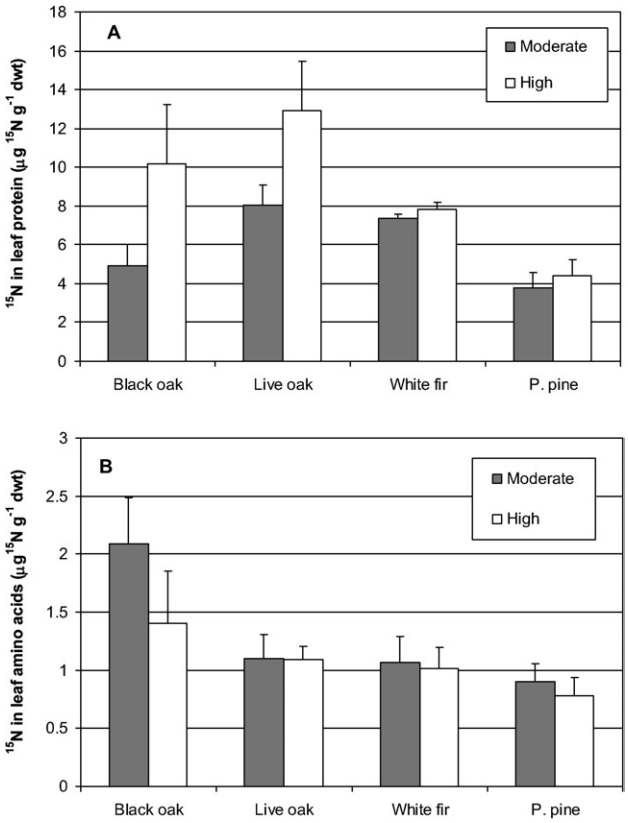

Fig. 3 Assimilation of dry deposition of nitric acid into leaf tissue. Panel A: ^{15}N incorporated into trichloroacetic acid precipitable proteins after 28 days of exposure to HNO_3 vapor. Panel B: ^{15}N incorporated into free amino acids after 28 days of exposure. ^{15}N content was determined by mass spectrometry. Data shown are the mean of 1 sample from 5 individuals, error bars = σ.

Foliar loading of dry deposited HNO_3. Even when uptake and assimilation are accounted for, total deposition to non-living, non-metabolically active foliage was substantially higher in most cases than total deposition to living foliage (Fig. 5). Total deposition measured as by ^{15}N retained added to N removed in

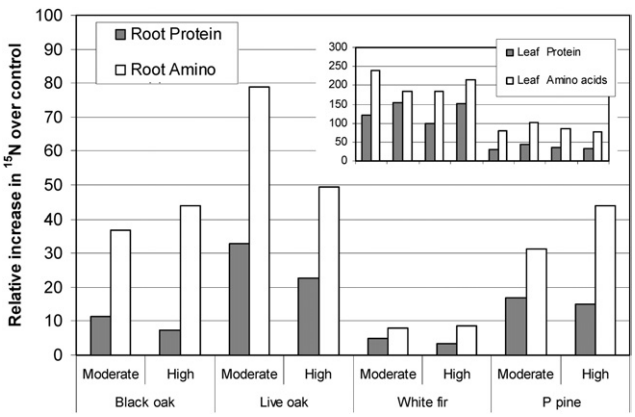

Fig. 4 Assimilation and translocation of dry deposition of nitric acid into root tissue. Data shown are relative ^{15}N abundance over control tissue because the extraction procedure was not quantitative. Inset shows the leaf ^{15}N data from Fig. 3 displayed as relative abundance over control for comparison to root incorporation data.

Table 3 Contribution of folar assimilation to the general nitrogen pool in foliage. Data shown are the mean ^{15}N content of 3 samples per individual and 5 individuals per treatment

		% leaf nitrogen labeled	
Treatment		Amino acids	Protein
Black oak	Low	1.055	0.524
	High	0.702	0.545
Live oak	Low	0.717	0.420
	High	0.802	0.641
White fir	Low	0.352	0.131
	High	0.412	0.161
Ponderosa pine	Low	0.352	0.158
	High	0.432	0.142

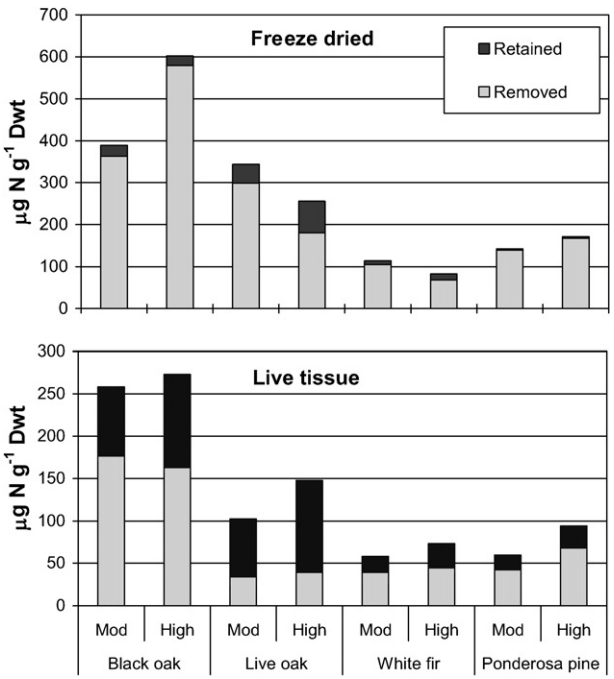

Fig. 5 Total HNO_3 deposition as measured by the amount of NO_3^- washed off of leaves, added to the amount of ^{15}N retained by leaves.

non-living black oak was more than twice the quantity measured in the living counterparts. Although most of the deposited HNO_3 was removed from the freeze-dried foliage, between 10 and 80 μg N g^{-1} dry weight was retained. In living foliage some of the retained ^{15}N was recovered in soluble protein and free amino acid pools (Fig. 3), but most of the retained ^{15}N was not in ether cellular component. Although uptake and assimilation of dry deposition is clearly a factor in canopy retention of the four species evaluated, only 10% to 20% of the retained N was recovered as assimilated N in the oak species and 30% to 60% of the retained N was recovered in the protein and AA pools in white fir and ponderosa pine were recovered in the assimilation pools (Table 3).

Discussion

These experiments focused on three physico-chemical and biological fates of dry deposition of HNO_3. (1) A quantitative assessment of the variability of dry deposition as measured by leaf washing among species under controlled conditions. (2) The influence of assimilation and non-assimilatory mechanisms of canopy retention. (3) The contribution of assimilation of deposited HNO_3 vapor is to the metabolic nitrogen pools of the four tree species.

Unit for calculating 'removal' 'retention' and 'deposition'

One of the more fundamental issues in comparing the four species was the selection of the appropriate units for deposition and retention. Comparing N removed on a *leaf area* basis gave one interpretation of the relationship among the four species, whereas comparisons of the same data as drawn on *dry weight* basis gave another. Using a simple one-sided leaf area typical of surface area calculations, the washable deposition collected from the freeze-dried foliage of white fir was lower that the other species, and it was slightly higher in ponderosa pine than the others. When the basis was shifted to a dry weight, both of the oaks species had N concentration removed significantly greater than either of the conifers. For estimates of dry deposition at the stand or landscape scale, surface area is important. Dry weight, on the other hand, is an important basis of comparison for biochemical activity. The differences are due to differences in leaf anatomy.[37] Black oak leaves have more spongy mesophyll with greater air space than do conifer needles, and the conifer needles have more densely packed palisade mesophyll cells, increasing the cellular density, than the oak leaves. In the interest of providing a dataset for the widest interpretation, we present both units where interpretations and conclusions were dependent on the unit of measure.

One of the difficulties in estimating dry deposition is the dependence of deposition velocity on micrometeorological conditions and surface characteristics.[38] Although the physics of gaseous deposition are well defined,[39] and many of the physico-chemical components of deposition are known, the biological activities of living organisms add a level of complexity that is poorly understood.[13,38,40] The comparison between living, metabolically active foliage, with foliage of similar physicochemical characteristics but lacking metabolic activity, provided insight into the importance of biological activity to estimating fluxes of HNO_3 dry deposition. In living foliage, the amount of washable NO_3^- reached a maximum foliar load of about 1.0 μg N cm^{-2} for black oak, live oak, and white fir and roughly 2 μg N cm^{-2} for ponderosa pine. The difference is largely a function of leaf geometry and leaf area measurements. In our experiments, leaf wash concentrations from living foliage reached a maximum after 14 d (perhaps earlier) under a high (137 ppb h), but realistic, dose. After an additional 14 d, no further accumulation was detected even though the applied dose doubled. Without the non-living samples for comparison, one might conclude that deposition rates as determined by leaf wash or throughfall were equivalent across species, although one could not quantify dry deposition above a dose of 137 ppb h.

The experimental evidence from the non-living specimens provided quite a different interpretation of dry deposition, however. In all but two cases, NO_3^- concentrations removed from non-living foliage were 3 to 6 times higher than that removed from their living counterparts. Moreover, N removed from foliage continued to increase with increasing exposure.

The soluble NO_3^- concentrations from freeze-dried samples collected after 28 d of were significantly higher that the 14 d samples for all four species

Microscopic observation indicated that freeze-drying did alter the surface slightly, but there is no reason to believe that the chemical composition of the cuticle surface was changed due to freeze-drying. However, the boundary layer conditions at the surface of a living leaf would be much different from that of a dead leaf. Under still conditions, a transpiring leaf would be surrounded by a layer of moist air as much as 3 mm thick; there may even be a layer of liquid water at the leaf surface.[41] In atmospheric studies it is well known that HNO_3 preferentially partitions into water vapor. In the presence of water vapor in the boundary layer, HNO_3 partitions into the aqueous phase. However, the maximum solubility of HNO_3 is 15.8 M, or 1.1 mol HNO_3 to 1.6 mol H_2O. A washable concentration of 1 μg N cm^{-2} HNO_3 dissolved in a saturated (vapor phase) boundary layer of 24.4 g H_2O m^{-3} would reach 16M in a vapor layer 0.03 mm deep. Possibly in theses experiments, all trees were all grown under identical environmental conditions and therefore had very similar boundary layer characteristics allowing them to reach a chemical saturation point within a similar timeframe.

In contrast, although the gaseous HNO_3 interacting with a dry, non-living surface would still involve a boundary layer of still air, but without transpiration, the water content in the boundary would reflect the ambient humidity of approximately 20% during the day during these experiments. We hypothesize that under these conditions solid phase reactions would dominate. Superficial dust, and the composition and reactivity of the epicuticular waxes would control deposition and retention. Unfortunately, very little is known regarding these types of reactions. Perhaps the interaction with soil particles as described in Padgett and Bytnerowicz[32] would provide a working model. Dry deposition of HNO_3 to sand, silt, and clay fractions varied with particle size, the smaller the particle, the more surface area was available, and the more NO_3^- was recovered. Under dry conditions deposition to soil particles did not show saturation at doses as high as 500 ppb h.

From an ecological perspective, it is interesting to note that although dry deposition to dead leaves was originally used as a means of understanding biological influences on dry deposition, the forests are huge repositories of dead leaves, and trees typically maintain leaves at all stages of the life cycle. Deciduous trees shed their leaves annually, and some like black oak have long abscission periods and will hold dead leaves in the canopy for several weeks (personal observation). Needles from ponderosa pine may persist for five to seven years, but every year the oldest whorl is shed, and like black oak, those dead needles may persist in the canopy for some time. The tendency for nonliving foliage to accumulate dry deposition at rates several times higher than living foliage, and the observation that a small, but significant portion of that deposition remains with the foliage poses some interesting questions regarding canopy retention, subsequent inputs into the ecosystem, and the interpretation of throughfall data.

Canopy retention and metabolic activity

After the leaves were washed, a portion of the deposited ^{15}N was retained in the tissue. That portion varied significantly across tree species when calculated on a dry weight basis. In all cases, the living foliage retained more of the deposited N than did the freeze-dried foliage, but all freeze-dried samples retained some ^{15}N. In the living samples, the retained ^{15}N was separated into the metabolic pools as determined by ^{15}N content in isolated free AA and soluble proteins and a 'non-metabolic pool'. For reference: on a dry weight basis leaf cells (aside from the cell wall) typically contain 50% protein (soluble and insoluble membrane proteins), 15% nucleic acids, 15% carbohydrates, 10% lipid and 10% 'other' which includes 0.5% chlorophyll and 0.3% free amino acids. Nitrogen is a component of all but carbohydrates and lipids.

Since the freeze-dried tissue did not have any metabolic activity, it is reasonable to assume that all retained ^{15}N was in the non-metabolic pool. In live oak, this portion was as much as 75 μg N g^{-1} Dwt retained in the non-metabolic pool. At the lower end, retention in ponderosa pine was less than 5 μg N g^{-1} Dwt, even though both species accumulated similar foliar loads of about 175 μg N g^{-1} Dwt. The mechanism for non-assimilatory retention is not known. It seems unlikely, given the solubility of HNO_3 in water and its insolubility in lipids or waxes, that retention was solely due to HNO_3 trapped in the cuticle. Nitric acid is a strong acid and a strong oxidant. The reactions between HNO_3 and organic molecules such as waxes and lipids are well described.[41–43] We hypothesize that the strong acid and oxidizing behavior of HNO_3 results in oxidation of the cuticular waxes and reduction of NO_3^- to a more lipophilic nitrogen oxide. Further investigation is underway.

Of the N retained by living tissue, the four species varied widely in the percent recovered in the metabolically active pools of proteins and AA. Black oak retained the greatest total amount of ^{15}N, but only 10% to 13% of the retained ^{15}N was recovered in AA or proteins, leaving 87% of the retained ^{15}N in either the non-metabolic pool, or in the smaller N-containing pools such as insoluble membrane proteins, chlorophyll, and nucleic acids. In contrast, white fir exposed to moderate HNO_3 concentrations retained the least total ^{15}N, but 62% was recovered in the AA or proteins pools. Although the total amount of total ^{15}N retained by ponderosa pine and white fir was much lower than that of the oaks, a much larger percentage was recovered in the metabolic pool.

The tendency to translocate assimilated N to root tissues was also species dependent. It is presumed that NO_3^- absorbed by foliage was assimilated in the shoots and translocated as AA to the roots, which provided the precursors for proteins that are synthesized in the roots.[44] However, we did not measure NO_3^- tissue content in these experiments as earlier experiments indicated tissue NO_3^- content was below detection limits. Ponderosa pine and black oak had similar levels of labeling in root tissue, whereas the ^{15}N recovered from white fir roots was only 5% to 8% greater than background. Live oak exported the greatest percentage of labeled N to roots. Both oak species had similar levels of ^{15}N label recovered in leaf tissue, approximately 300% excess abundance, but live oak exported substantially more ^{15}N, at 70% to 120% excess abundance, as compared to an excess abundance of 50% in black oak.

Some of the unrecovered ^{15}N may have been in nucleic acids, chlorophyll or secondary metabolites, and some free NO_3^- was most likely in the apoplastic or symplastic space, but it is unlikely that these molecules could account for 85% of the missing ^{15}N in

black oak for example. The ^{15}N not recovered in the biologically active pools in combination with the ^{15}N retained by non-metabolically active tissue indicates that uptake and assimilation does not fully explain canopy consumption.

Deposition contribution to nitrogen status of trees

Of the N that was taken up and assimilated does 1% of the AA or less than 0.5% or the proteins represent a significant contribution to the overall N status of these trees? Vose and Swank,[24] who arrived at similar numbers using different techniques, argued this was not significant, but Boyce *et al.*,[23] who investigated uptake of wet deposition argued to the contrary. We tend to agree with the arguments of Boyce *et al.*[23] that short-term assimilation resulting in of 1% of the AA pool is indeed significant. Nitrogen is very well conserved in perennial plants and uptake reflects demand.[45] As Nussbaum *et al.*[19] pointed out, different growth stages have different requirements for nitrogen, but extrapolating short-term exposures to long-term responses should be approached cautiously. In these experiments, all trees had completed their annual growth phase; therefore, N demand was low. Obviously, the next step would be to examine foliar uptake when N demand is high. Dry deposition in Mediterranean climates can occur year round. Keeping in mind the caveats of over-extrapolating short term experiments, if these trees captured 1% of their nitrogen needs every month for one year, by the end of that period, 12% of the metabolically active nitrogen would have been acquired from atmospheric deposition of anthropogenic air pollution. Multiply that over a decade of exposure and 1% in 28 d can become a significant contribution to the cellular nitrogen pools. However, direct extrapolation of controlled environment experiments to natural ecosystems should be approached cautiously.[49]

Perhaps uptake and absorption of dry deposited N represents a wind-fall for the forest, but perhaps not. It is now pretty well established that export and translocation of free amino acids are a signaling mechanism between shoots and roots. When the shoots have sufficient N to maintain tissues and growth, the excess may be retranslocated to the roots in phloem, theoretically signaling root cortical cells that the N needs of the shoot are met. Rennenberg *et al.*[46] speculated that even small amounts of foliar assimilated dry deposition exported to the roots would trigger roots to reduce uptake of soil-available N. Without trees to contribute to the uptake and sequestering of excess N in soils, the effects of atmospheric deposition on the forest floor ecology and hydrology becomes even more dramatic. Moreover, N abundance has the well described effect of reducing root growth in favor shoot growth.[47] Smaller root systems mean less surface area for water uptake and uptake of other nutrients. Although the direct effect of foliar loading and metabolic assimilation of dry deposited HNO_3 on these and other ecological processes have not been proven, clearly foliar uptake has the potential for perturbing and disrupting the normal functions in trees and forest stands.

Conclusions

Dry deposition of HNO_3 on living leaves is largely dependent upon boundary layer conditions, particularly the water vapor density at the leaf surface.[28] Under controlled environmental conditions, foliar loading to living leaves was remarkable consistent across the four species; all four species evaluated exhibited a saturation point where continued exposure did not result in increased N removed in wash solutions. Dry deposition to freeze dried leaves appeared to be controlled more by surface properties rather than aqueous boundary layer conditions. Foliar loading was more variable in the freeze-dried samples across species and consistently higher than their living counterparts.

Fate of deposited HNO_3 fell into three categories: (1) readily soluble, which was removed by leaf wash, (2) retained and assimilated into the metabolic nitrogen pools, and (3) retained, but apparently not assimilated into biologically active molecules. The percentage of dry deposition assimilated was low, but if the quantities assimilated after one month are extrapolated to several years, the deposition represents a significant contribution to the N pools of plants. The retained fraction not recovered as AA or proteins is a bit of a mystery. No doubt some N is in metabolic pools not measured, such as chlorophyll and nucleic acids. But given the known distribution of N-containing compounds in plants most of the missing retained ^{15}N was likely in a non-metabolically active pool.

Most trees contain leaves at every stage of the growth cycle during the growing season; therefore, at every variable in boundary layer condition and metabolic N requirement. At the moment, canopy interaction models and inferential measurements do not capture those variables. We argue that foliar receptors, though ecologically relevant, are not very good passive collectors for estimates of atmospheric deposition because of variability in biological activity in trees. But a better understanding of the biophysical and biochemical interactions between dry deposition and plants will improve our ability to quantify this critical component of atmospheric deposition.

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